The role of norepinephrine and alpha-adrenergic receptors in acute stress-induced changes in granulocytes and monocytes

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Abstract: OBJECTIVE: Acute stress induces redistribution of circulating leucocytes in humans. Whereas effects on lymphocytes as adaptive immune cells are well-understood, the mechanisms underlying stress effects on granulocytes and monocytes as innate immune blood cells are still elusive. We investigated whether the stress hormone norepinephrine (NE) and -adrenergic receptors (-ADRs) may play a mediating role. METHODS: In a stress study, we cross-sectionally tested in 44 healthy men for associations between stress-induced NE increases and simultaneous granulocyte and monocyte cell count increases, as measured immediately before and several times after the Trier Social Stress Test. In a subsequent infusion study, 21 healthy men participated in three different experimental trials with sequential infusions of 1 and 15-min duration with varying substances [saline as placebo, the non-specific -ADR blocker phentolamine (2.5mg/min), and NE (5 g/min)]: trial1=saline+saline, trial2=saline+NE, trial3=phentolamine+NE. Granulocyte and monocyte cell numbers were assessed before, immediately after, 10min, and 30min after infusion procedures. RESULTS: In the stress study, higher NE related to higher neutrophil stress changes (=.31,p=.045, Rchange=.09), but not epinephrine stress changes. In the infusion study, saline+NE induced significant increases in neutrophil (F(3/60)=43.50,p<.001, =.69) and monocyte (F(3/60)=18.56,p<.001, =.48) numbers compared to saline+saline. With phentolamine+NE, neutrophil (F(3/60)=14.41,p<.001, =.42) and monocyte counts (F(2.23/44.6)=4.32,p=.016, =.18) remained increased compared to saline+saline but were lower compared to saline+NE (neutrophils: F(3/60)=19.55,p<.001, = monocytes: F(3/60)=2.54,p=.065, =.11) indicating partial mediation by -ADRs. Trials did not differ in eosinophil and basophil count reactivity. CONCLUSIONS: Our findings suggest that NE-induced immediate increases in neutrophil and monocyte numbers resemble psychosocial stress effects and can be reduced by blockade of -ADRs.

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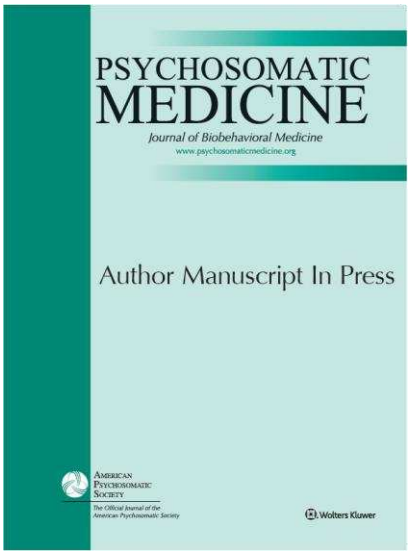

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The role of norepinephrine and alpha-adrenergic receptors in acute stress-induced changes in granulocytes and monocytes

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ABSTRACT

Objective: Acute stress induces redistribution of circulating leucocytes in humans. Whereas effects on lymphocytes as adaptive immune cells are well-understood, the mechanisms underlying stress effects on granulocytes and monocytes as innate immune blood cells are still elusive. We investigated whether the stress hormone norepinephrine (NE) and α-adrenergic receptors (α-ADRs) may play a mediating role.

Methods: In a stress study, we cross-sectionally tested in 44 healthy men for associations between stress-induced NE increases and simultaneous granulocyte and monocyte cell count increases, as measured immediately before and several times after the Trier Social Stress Test. In a subsequent infusion study, 21 healthy men participated in three different experimental trials with sequential infusions of 1 and 15-min duration with varying substances [saline as placebo, the non-specific α-ADR blocker phentolamine (2.5mg/min), and NE (5µg/min)]: trial1=saline+saline, trial2=saline+NE, trial3=phentolamine+NE. Granulocyte and monocyte cell numbers were assessed before, immediately after, 10min, and 30min after infusion procedures.

Results: In the stress study, higher NE related to higher neutrophil stress changes (β=.31,p=.045, R^2change=.09), but not epinephrine stress changes. In the infusion study, saline+NE induced significant increases in neutrophil (F(3/60)=43.50,p<.001,η^2=.69) and monocyte (F(3/60)=18.56,p<.001,η^2=.48) numbers compared to saline+saline. With phentolamine+NE, neutrophil (F(3/60)=14.41,p<.001,η^2=.42) and monocyte counts (F(2.23/44.6)=4.32,p=.016,η^2=.18) remained increased compared to saline+saline but were lower compared to saline+NE (neutrophils: F(3/60)=19.55,p<.001,η^2=.494, monocytes: F(3/60)=2.54,p=.065,η^2=.11) indicating partial mediation by α-ADRs. Trials did not differ in eosinophil and basophil count reactivity.

Conclusions: Our findings suggest that NE-induced immediate increases in neutrophil and monocyte numbers resemble psychosocial stress effects and can be reduced by blockade of α-ADRs.

Keywords: granulocytes, monocytes, norepinephrine, phentolamine, alpha-adrenergic receptor blocker, stress

Abbreviations:

ADR, adrenergic receptor

BP, blood pressure

BMI, body mass index

DBP, diastolic BP

EPI, epinephrine

HPA, hypothalamus-pituitary-adrenal axis

HPLC, high-pressure liquid chromatography

i.v., intravenous

MAP, mean arterial blood pressure

NE, norepinephrine

SAM, sympathetic adrenomedullary system

s.c., subcutaneous

SBP, systolic BP

SEM, standard error of the mean

t1/2, half-life time

TSST, Trier Social Stress Test

1. Introduction

Acute psychosocial stress induces a variety of physiological reactions preparing an organism to perform fight-or-flight reactions and to cope with potential consequences, such as, injuries and related tissue damage (1). Physiological stress reactivity comprises activation of the two main stress axes: the first-wave (or immediate) sympathetic adrenomedullary (SAM) system with release of its stress hormones epinephrine (EPI) and norepinephrine (NE), and the second-wave (or delayed) hypothalamus-pituitary-adrenal (HPA) axis (1). Stress-responsive physiological reactions that prepare an organism to cope with potential tissue damage and related infections range from increased procoagulant activity (2) to immune system activation, including leukocyte redistribution (3). A variety of studies clearly demonstrate that acute stress induces the redistribution of lymphocytes as blood cells of the adaptive immune system (4-6). So far, studies investigating stress-responses of innate immune cells and underlying mechanisms have extensively focused on natural killer (NK) cells (7-9), whereas comparably little is known about other innate immune cells.

Prevalent blood cells of the innate immune system's immediate and unspecific defenses comprise of monocytes and granulocytes having the three subtypes neutrophil, eosinophil, and basophil granulocytes. Studies investigating acute psychosocial stress effects on these innate myeloid immune cells in healthy participants most consistently report transient immediate increases, particularly in monocyte and neutrophil numbers (10-17). Some studies still found increases in monocyte (13, 16) and granulocyte (13, 18, 19) numbers 10-15 min after stress cessation, whereas the majority of studies failed to detect monocyte (10, 11, 18-20) and granulocyte (10, 11, 20) increases at this and later time-points up to 120 min (10, 11, 19-21). From an evolutionary "fight-flight" perspective, an acute stress-situation (e.g. imagine a hunter

trying to kill a dangerous animal) implicated the risk of being injured. According to this, acute stress-induced transient immediate increases in circulating numbers of monocytes and neutrophils in particular may represent an evolutionary founded reaction that prepares for potential tissue damage and related infection. The main effector functions of monocytes and granulocytes are crucial for survival and include the phagocytosis of pathogens, antigen-presentation, and the production and release of different mediators such as cytokines. The latter coordinate subsequent immune reactions, but also regulate reactive oxygen species and proteolytic enzymes that disintegrate tissue surfaces (22-25). In the context of repeated or chronic stress, however, repetitive activation of the innate immune response with sustained cytokine production may no longer be adaptive. Based on the model of allostatic load (26), it can indeed give raise to a systemic low-grade inflammatory state with many adverse health consequences, including cardiovascular disease (e.g. (27)).

Hitherto, the mechanisms underlying immediate acute stress-induced increases in monocyte and granulocyte cell numbers are not fully understood. There is evidence that catecholamine release may play a mediating role. On the one hand, EPI and NE increases following stress and monocyte and granulocyte increases peak at the same time, i.e. immediately after stress (e.g. (10, 17, 28)). However, cross-sectional studies that relate stress-induced catecholamine release and monocyte/granulocyte cell count changes are scarce. To the best of our knowledge, only one cross-sectional study investigated associations in women between plasma EPI levels and granulocyte counts in reaction to a speech task at peak reactivity, i.e. immediately after stress, and found a positive correlation (29). Yet, infusion studies consistently demonstrate immediate granulocyte (in particular neutrophil) and / or monocyte increases in reaction to infusion or injection of either EPI (30-35), or NE in humans (30, 36). It is important to note, that *in vivo*

infusion of EPI can dose-dependently induce increases of both EPI and NE (7, 35, 37), whereas NE infusion increases NE only (7, 38). Thus, it is possible that the observed cell increases following EPI infusion are (co)-induced by NE. Notably, infusion duration in these studies ranged from 30 min to 2 h. This was more than twice as long as typical stress tests and the total amount of substance infused was within the supraphysiological range (30, 31, 36) applied in all NE and most EPI infusion studies. As yet, there is a lack of infusion studies applying a design that mimics reactivity to typical standardized psychological stress tests in terms of both infusion dosage and duration.

The receptor mechanisms for catecholamine induced redistribution of monocytes and granulocytes are not clear either. NE and EPI have different affinities to α- and β-adrenergic receptors (ADRs; (39)). So far, most studies investigated β-ADR mechanisms and in sum found β-ADR to play a partial role, but to not account for full mediation of monocyte and granulocyte cell number increases (for details see Text, Supplemental Digital Content 1, http://links.lww.com/PSYMED/A492). Here, the involvement of α-ADR is a likely explanation. Human studies investigating α-ADR mechanisms are lacking, but two animal studies suggest a mediating role at least for granulocyte increases: Acute α-ADR blockade by phentolamine neutralized NE-infusion induced splenic granulocyte release in guinea pigs (40). Likewise, 12 h pre-treatment with phentolamine prevented rats from social defeat stress and induced increases in circulating and splenic granulocytes, but failed to inhibit stress-induced monocyte increases (41). Taken together, evidence suggests that catecholamines are involved in the mediation of stress-induced transient increases in innate immune blood cell numbers, especially of granulocytes. However, with respect to underlying mechanisms, the specific roles of the catecholamine NE and α-ADR (42, 43) remain unclear, particularly in humans.

In this study, we examined for the first time in healthy men cross-sectional associations of stress-induced NE and EPI increases with increases in granulocyte and monocyte counts. Since we found the stress-induced NE-increase to relate to neutrophil increases, we further investigated causality of this finding with a placebo-controlled *in vivo* infusion experiment. We infused in healthy men a dosage of NE that mimics NE-stress reactivity effects in terms of blood pressure increases as typically observed in reaction to standardized stress induction by the widely used Trier Social Stress Test (TSST, (38, 44, 45)). The infusion duration corresponded to that of the TSST and NE infusion and was performed with and without preceding specific α-ADR blockade by phentolamine to identify a potential involvement of α-ADR mechanisms. We hypothesized that stress-induced catecholamine increases would relate to simultaneous innate immune cell count increases, in particular of neutrophils and that our NE infusion would induce transient immediate increases in monocyte and neutrophil counts. Furthermore, that prior phentolamine application would reduce these increases.

2. Methods

2.1 Participants

Here, we present data from two *in vivo* studies in humans, a *stress study* and a subsequent *infusion study*. Both studies were parts of larger projects and, in addition to participants' characteristics, the catecholamine data of both studies, as well as verification of successful phentolamine administration of the infusion study, were previously reported (38, 46).

Participants of both studies comprised medication-free, non-smoking healthy white men between 21 and 66 years of age. We recruited men to rule out potential confounding influences by the menstrual cycle (47-49). Specific exclusion criteria were verified by telephone screening (stress

study) or a medical interview (infusion study) and included: smoking; any regular or acute medication intake; illicit drug abuse; psychiatric disorders; any heart disease, varicosis or thrombotic diseases; metabolic (e.g. diabetes, elevated cholesterol levels), liver, renal, or pulmonary diseases; rheumatic diseases; cancer; allergies and atopic diathesis; HIV-infection; and any current infections. All participants provided written informed consent prior to study begin. Both studies were carried out in accordance with the Declaration of Helsinki principles and were approved by the Ethics Committees of the Cantons of Zurich (stress study) and Bern (infusion study), respectively. The Swiss Agency for Therapeutic Products (Swissmedic) formally approved the research protocol of the infusion study. Participants of the infusion study were compensated with 120 CHF per day (360 CHF for all three days), and participants of the stress study were paid 80 CHF.

The stress study comprised of 44 participants with complete blood count data (data collection from April until October 2004) who were recruited through advertisement at the University Zürich and with the help of the Swiss Red Cross (Zurich). Participants of the infusion study were recruited with the aid of the Swiss Red Cross (Bern) and the Clinical Investigation Unit of the University Hospital of Bern (Inselspital). Twenty-one normotensive participants with complete blood count data finished all three trials of the infusion study with a fully balanced trial sequence, rendering a total of 63 trials (data collection from August 2009 until May 2012).

2.2 Design and procedure

Stress study

In the first study, participants were instructed to avoid alcohol, caffeinated beverages and strenuous physical exercise as from the evening before reporting to our lab at the University of

Zurich between 1400 and 1600 h. After catheter insertion into the brachial vein of the non-dominant arm, followed by a 45 min resting interval in a quiet room, acute psychosocial stress was induced using the standard protocol of the Trier Social Stress Test (TSST; (44)). After a short introduction, participants underwent a 5-min preparation phase followed by a 5-min mock job interview, and a 5-min mental arithmetic task in front of a video camera and a committee. After the TSST, participants remained seated in a quiet room. Blood samples were collected immediately before participants were introduced to the TSST, immediately after completion of the TSST, as well as 20 and 60 min afterwards.

Infusion study

The infusion protocol is based on the 15-min TSST paradigm and aims to investigate effects of a NE-infusion. It was designed to mimic NE-stress-reactivity with and without α-adrenergic blockade. The study was performed at the Clinical Investigation Unit of the Bern University Hospital (Inselspital). In a single-blind, placebo-controlled, within subject design all participants took part in three different experimental trials varying in terms of the combination of two sequentially infused substances as previously described (38). These were saline+saline in trial 1, saline+NE in trial 2, and phentolamine+NE in trial 3. Trial 1 was the placebo condition and was performed to control for potential effects of the infusion procedure per se. Trial 2 was the main experimental trial designed to test for the effects of NE infusion. Trial 3 tested whether potential NE effects would be modulated by α1- plus α2-ADR, as phentolamine is a non-specific α-ADR blocking compound.

The trial-sequence was fully counterbalanced by using a Latin Square design with the sequences: 1,2,3 (i.e. infusion day 1 was trial-1, infusion day 2 was trial-2, infusion day 3 was trial-3); 2,3,1

(i.e., infusion day 1 was trial 2, infusion day 2 was trial 3, infusion day 3 was trial 1); and 3,1,2 (i.e., infusion day 1 was trial 3, infusion day 2 was trial 1, and infusion day 3 was trial 2). Trials took place on separate days with inter-trial intervals of at least one week to two weeks, to allow for phentolamine wash-out. Ethical and safety considerations regarding potential (hemodynamic) side effects of study substances prohibited a double-blind design. Therefore, the participants, but not the experimenters, were blind to trial substances. All infusions were performed by a board-certified internist.

Similar to the stress study, participants abstained from alcohol and caffeinated beverages as from the evening before the test day. Moreover, they abstained from physical exercise for 24h and maintained a regular sleep-wake rhythm during the three nights before each trial, with lights out between 22:30h and 24:00h and lights on between 07:00h and 09:00h. Participants reported to the lab at 11:45h to receive a standardized meal with experimental procedures starting at 1300h. Participants were tested in supine position lying on a bed. Each trial started with a 10-min introduction phase during which the testing procedure was explained, followed by catheter insertion into the brachial vein of the dominant arm for the infusions. For blood sampling a second catheter was inserted into the brachial vein of the non-dominant-arm. After a further 45-min interval to acclimatize, the infusion procedure started.

For the assessment of blood cell counts, we focused on the fast kinetics of the acute blood cell response identified in the stress study, and collected blood samples immediately before the first infusion (baseline), as well as 1, 10, and 30 min after the infusion procedure ended. Blood samples for NE and EPI assessments were taken at baseline before the first infusion and 1 min after the second infusion. Resting blood pressure was measured twice during the medical interview prior to study begin. As previously described in detail (38), we demonstrated effective

phentolamine application in this study by BP and heart rate assessment using Omron sphygmomanometry (Omron 773, Omron Healthcare Europe B.V. Hoofdorp, Netherlands).

2.3 Substance infusion

In the infusion study, we applied two sequential infusions with application of either saline or phentolamine for 1 min (first infusion), and NE or saline for 15 min (second infusion), with an interval of 5 min between infusions. The post-infusion phase began after the second infusion.

To mimic NE stress reactivity effects, NE (Sintetica, SA, Mendrisio, Switzerland) was diluted in saline and the resulting solution of 5µg/ml was infused with 1 ml/min over 15 min. In total, 75 µg NE was administered. We chose a 15-min infusion time for NE to parallel the duration of the TSST.

The non-selective α-adrenergic antagonist (i.e., α1- and α2-AR-blocker) phentolamine (Regitin®, Novartis Pharma AG, Basel, Switzerland) was diluted in saline and 5ml of 0.5mg/ml, totaling 2.5mg phentolamine, was infused within 1 min. Identical times of 1 min and 15 min were used for saline infusions (38).

2.4 Physiological measurements

Venous blood was drawn in EDTA-coated monovettes (Sarstedt, Numbrecht, Germany).

To assess granulocyte and monocyte numbers, as well as hematocrit and hemoglobin concentrations, 5-part differential blood cell counts were obtained on automated hematology systems in the University Center for Laboratory Medicine and Pathology of the University Hospital Zurich (stress study: Advia 120, Bayer Diagnostics) and the University Institute of Clinical Chemistry of the Bern University Hospital (infusion study: Advia 120, Siemens

Diagnostics). Notably, hematocrit and hemoglobin were assessed in order to control for confounding effects of potential stress- or infusion-induced hemoconcentration, i.e. plasma volume changes following previous methods (50).

For NE and EPI assessment, blood was immediately centrifuged for 10 min at 2000g and 4°C; plasma was stored at -80°C until analyzed. Plasma NE and EPI levels were determined by means of high-pressure liquid chromatography (HPLC) using electrochemical detection after liquid-liquid extraction in the Laboratory of Stress Monitoring, Göttingen, Germany (51, 52). The lower limit of a detection was 12 pg/ml each for EPI and NE; inter- and intra-assay CVs were <5%. Undetectable EPI values were replaced by half the detection limit. In two participants of the infusion study, some NE and EPI levels were missing due to technical problems with HPLC.

2.5 Statistical analyses

Data was analyzed using SPSS (version 23.0) statistical software package (SPSS Inc., Chicago IL, USA) and presented as mean±SEM (if not indicated otherwise). All tests were two-tailed with a level of significance of $p \leq .05$. Before analyses, all raw data were examined for normal distribution using the Kolmogorov-Smirnov test. As some cell count data was skewed, all cell count raw data was logarithmically transformed for the analyses, but the original data is presented in the Tables and Figures for clarity.

We calculated body mass index (BMI) as the weight in kilograms divided by the square of the height. Mean arterial blood pressure (MAP) was calculated from resting BP measurements using fully automated sphygmomanometry devices (Omron 773, Omron Healthcare Europe B.V. Hoofdorp, The Netherlands) using the formula 2/3 mean diastolic blood pressure (DBP) + 1/3 mean systolic BP (SBP). Manipulation-induced changes in NE (NE change), EPI (EPI change),

blood cell count (blood cell count change), and plasma volume percentage (plasma volume percentage change) were calculated as the difference in plasma levels between the peak, i.e. 1 min post experimental manipulation (i.e. stress or infusion) and the respective baseline (i.e. before manipulation).

Stress study: To test for stress effects on blood cell counts, catecholamines, and plasma volume, we calculated general linear models with repeated measurement. Associations between stress-induced catecholamine and blood cell count changes were calculated using linear regression analyses with EPI and NE stress change scores as independent variables controlling for age, BMI, and MAP. We controlled for these potential confounding variables because (a) of well-known age effects of stress on the sympathetic nervous system and the immune system (53), because (b) BMI was found to relate to neutrophil baseline counts (54) and altered stress-induced cytokine secretion by stimulated immune cells (46), and because (c) catecholamine stress reactivity was found to be higher in hypertensives (45).

Infusion study: To test for differences between infusion-trials in *baseline* NE and EPI concentrations, as well as blood cell counts, we calculated general linear models with repeated measurement, with the baseline levels of each trial as repeated dependent variables, and report post-hoc tests to identify differences between trials (i.e. trial-1 vs. 2, trial-1 vs. 3, and trial-2 vs. 3). Trial differences in NE and EPI changes, as well as plasma volume changes, were calculated accordingly.

To test the effects of the different infusion trials on *blood cell counts over time*, we compared trials pairwise (i.e. trial-1 vs. 2, trial-1 vs. 3, and trial-2 vs. 3) by calculating general linear models with the two repeated factors trial (2 trials) and time (4 blood cell count time-points). Moreover, we tested post-hoc whether blood cell counts, catecholamines, and plasma volumes

significantly changed over time in each trial by calculating general linear models with repeated measures in each trial separately. Post hoc tests comprised further of paired *t*-tests to test for differences between baseline and the expected peak, i.e. 1 min after infusions in each trial.

All blood cell count analyses are presented without and with adjustment for plasma volume changes following the respective experimental manipulation according to previous methods (50). Effect size parameters (*f*) were calculated from partial η^2-values and are reported where appropriate (effect size conventions: *f*: .10=small, .25=medium, .40=large).

3. Results

3.1 Participants' characteristics

As depicted in Table 1, study participants were men varying in age, BMI and MAP.

3.2 Baseline measures and treatment-induced changes in catecholamines and plasma volume

Table 2 summarizes baseline cell counts in the two studies and treatment-induced changes of both catecholamine concentrations and plasma volume percentages.

Stress study

Stress-induced changes: As previously described (55), stress induced significant increases in NE ($F(1/43)=136.57$, $p<.001$, $f=1.78$, $\eta^2=.76$) and EPI ($F(1/43)=22.68$, $p<.001$, $f=0.73$, $\eta^2=.35$) levels from baseline to post stress (see Figure S1, Supplemental Digital Content 2, http://links.lww.com/PSYMED/A493). Plasma volume decreased in reaction to stress

provocation (F(2.75/118.21)=49.90, p<.001, f=1.08, η^2=.54 with lower values immediately after stress that recovered quickly.

Infusion study

Baseline measures: As shown in Table 2, there were no baseline (i.e. pre-infusion) differences between the three trials in terms of granulocyte and monocyte numbers, and plasma catecholamines (p's≥.17).

Infusion-induced changes: Both saline+NE and phentolamine+NE led to increased NE levels compared with saline+saline (p´s<.001), whereas NE changes did not differ between saline+NE and phentolamine+NE (p=.79). Saline+NE led to decreased EPI levels compared with saline+saline (p=.013) and phentolamine+NE (p=.027), whereas EPI changes were similar between saline+saline and phentolamine+NE (p=.75). Comparisons between trials showed significant differences in infusion-related plasma volume percentage changes (F(2/40)=24.063, p<.001, f=1.10, η^2=.55) between saline+NE and saline+saline (p<.001), between phentolamine+NE and saline+saline (p<.001), as well as between saline+NE and phentolamine+NE (p=.005). Both saline+NE (F(2.73/54.61)=22.63, p<.001, f=1.06, η^2=.53) and phentolamine+NE (F(2.84/56.75)=21.37, p<.001, f=1.03, η^2=.52) led to decreased plasma volume over time; saline+saline however increased plasma volume over time (F(1.9/38.0)=5.08, p=.012, f=0.50, η^2=.20).

3.3 Stress study: Blood cell count reactivity to stress and associations with catecholamine stress responses

Stress induced significant increases in all granulocyte types (neutrophils: F(2.94/126.29)=23.04, p<.001, f=0.73, η^2=.35; eosinophils: F(2.45/105.32)=3.75, p=.019, f=0.29, η^2=.08; basophils:

F(2.57/110.51)=3.47, p=.024, f=0.28, η^2=.08), and monocytes (F(3.00/129.00)=29.18, p<.001, f=0.82, η^2=.40) with highest cell counts immediately after stress (see Figure 1). Correcting for hemoconcentration did not significantly change results for neutrophils and monocytes (p's <.001), but basophil increases became of borderline significance (p=.079) and eosinophil increments were no longer significant (p=.74).

Linear regression analyses revealed that independent of age, BMI, and MAP, higher NE, but not EPI, stress changes related to higher neutrophil stress changes without (NE: β=.31, p=.045, R^2 change=.09; EPI: p=.12), and with correction for hemoconcentration (NE: β=.32, p=.047, R^2 change=.10; EPI: p=.45). Neither NE (p´s>.77) nor EPI (p´s>.27) significantly related to stress changes of monocytes, basophils, or eosinophils.

3.4 Infusion study: Blood cell count changes in reaction to infusions in the trial conditions

As compared to saline+saline, significant increases over time in neutrophil and monocyte numbers were induced both by saline+NE (neutrophils: F(3.00/60.00)=43.50, p<.001, f=1,47, η^2=.69; monocytes: F(3.00/60.00)=18.56, p<.001, f=.96, η^2=.48) and phentolamine+NE (neutrophils: F(3.00/60.00)=14.41, p<.001, f=.85, η^2=.42; monocyte: F(2.23/44.61)=4.32, p=.016, f=.47, η^2=.18). Correction for hemoconcentration did not significantly change results except that monocyte differences between phentolamine+NE and saline+saline became of borderline significance (p=.056). Phentolamine+NE induced lower neutrophil increases compared to saline+NE (F(3.00/60.00)=19.55, p<.001, f=0.99, η^2=.49; with correction for hemoconcentration: p<.001) and also induced lower monocyte increases, which were notably of borderline significance (F(3.00/59.96)=2.54, p=.065, f=0.36, η^2=.11; with correction for hemoconcentration: p=.14).

There were no significant trial differences in eosinophil and basophil counts over all time-points, either without (p´$s \geq .11$) or with correction for hemoconcentration (p´$s > .13$) (see Figure 2).

Post hoc analyses in each trial confirmed significant neutrophil and monocyte increases over time with saline+NE (neutrophils: $F(3/60)=70.74$, $p < .001$, $f=1.88$, $\eta^2=.78$; monocytes: $F(2.94/58.77)=15.870$, $p < .001$, $f=0.89$, $\eta^2=.44$) and phentolamine+NE (neutrophils: $F(2.73/54.52)=31.15$, $p < .001$, $f=1.25$, $\eta^2=.61$; monocytes: $F(2.21/44.16)=6.91$, $p=.002$, $f=.59$, $\eta^2=26$). Correction for hemoconcentration did not significantly change these results (p´s $< .007$). Moreover, saline+NE, but not phentolamine+NE, induced significant increases in eosinophil numbers ($F(2.02/40.46)=3.32$, $p=.046$, $f=0.41$, $\eta^2=.14$; trial 3: $p=.42$), although not independent of hemoconcentration (p's$>.093$). Basophil counts however, did not significantly change with saline+NE and phentolamine+NE (p´$s > .14$). Saline+saline decreased neutrophil counts over time ($F(2.58/51.51)=14.47$, $p < .001$, $f=0.85$, $\eta^2=.42$) independently of hemoconcentration ($p=.002$), whereas no other cell type changed significantly (p´$s > .25$).

Paired t-tests revealed that saline+NE induced significant increases in numbers of all cell types from baseline immediately after the infusion procedure (monocytes: $t(20)=9.06$, $p < .001$; neutrophils: $t(20)=9.58$, $p < .001$; eosinophils: $t(20)=2.68$, $p=.014$; basophils: $t(20)=2.13$, $p=.046$). Changes in monocytes, neutrophils, and eosinophils were independent of hemoconcentration (p´$s < .022$), whereas basophil changes became of borderline significance ($p=.076$) after correction for hemoconcentration. Phentolamine+NE induced increases in monocytes ($t(20)=2.95$, $p=.008$) and neutrophils ($t(20)=2.26$, $p=.035$), but not eosinophils ($p=.27$) or basophils ($p=.49$). Only the increase in monocyte counts was independent of hemoconcentration ($p=.011$). Saline+saline reduced neutrophil counts ($t(20)=-3.08$, $p=.006$), although not independently of hemoconcentration ($p=.13$), but reduced no other cell type counts (p´$s \geq .13$).

Supplemental Text in Supplemental Digital Content 3, http://links.lww.com/PSYMED/A494, describes associations of age, body mass index (BMI), and mean arterial blood pressure (MAP) with basal and reactivity cell counts.

4. Discussion

Here, for the first time we present data combining human cross-sectional assessment with an infusion paradigm in order to shed light on the mechanisms underlying stress-induced increases in innate immune cell numbers.

4.1 Stress study

In response to psychosocial stress, we found immediate transient increases in neutrophil and monocyte numbers with and without plasma volume change correction. Notably, we corrected for hemoconcentration since changes in plasma volume can confound cell count measurements (56). Our observation is in line with other studies showing immediate increases of neutrophils and monocytes after various acute stress paradigms (10-17). The observed small-scale increases of eosinophil and basophil cell counts following stress were in line with observations in atopic dermatitis patients (10), but became insignificant after hemoconcentration correction.

The main finding of our stress study was that stress-induced plasma NE concentration increases related to the observed transient elevation of neutrophil cell counts immediately after stress cessation, but not thereafter. We were unable to find a second response of neutrophils, as has been reported one hour after stress cessation (3, 14, 18). The quick start and end of the stress-induced release of catecholamines (57) from both, adrenal medulla and sympathetic nerve endings and their short half-life time in blood (e.g. $t_{1/2}$(NE) = 2.5 min in humans, (58)) may

explain this kinetic during, and immediately after the stress test. In a previous study in young women, increments in EPI but not NE concentrations were associated with the total granulocyte numbers immediately after psychosocial stress, although this association vanished after hemoconcentration correction (29). Reasons for this divergence may relate to differences in age and gender and the intensity and duration of stress induction paradigms between studies.

4.2 Infusion study

In the second study we infused an amount of NE (trial-2, 75µg) in a saline-controlled (trial-1) paradigm, which mimics the physiological stress response to the TSST in terms of NE release duration (15 min (57)), plasma volume changes (-3.4% (59)), and blood pressure increases (38). Before any administration, neither catecholamine plasma levels nor cell counts differed between trials, so a randomization bias could be excluded.

With respect to innate immune cell numbers, we observed an increase of neutrophil granulocytes and monocytes immediately after NE infusion that was reduced by prior phentolamine application, particularly in neutrophils. Statistical correction for plasma volume changes confirmed increased neutrophil numbers following NE infusion with and without α-ADR blockade. Whereas significant differences for monocyte dynamics remained only after NE infusion without α-ADR blockade. Moreover, eosinophil but not basophil counts increased following NE infusion without α-ADR blockade, however, not independently of hemoconcentration. These results suggest that comparable with other *in vivo* NE infusion studies, we could validate an immediate NE-induced increase of monocytes (36) and neutrophils (30, 36). Also, *in vivo* EPI infusions led to immediate transient increases of monocyte and neutrophil

cell counts (30-33, 35, 60). In line with other stress (10) and infusion (33) studies in healthy participants, we could not observe independent increases in eosinophil and / or basophil counts.

4.3 Underlying mechanisms

With respect to underlying *ADR mechanisms*, our data suggests an inhibiting effect of α-ADR blockade by phentolamine on NE-induced mobilization of neutrophils and monocytes, and that the phentolamine effect on monocytes but not on neutrophils did depend on hemoconcentration. To the best of our knowledge, no other study hitherto investigated effects of catecholamine infusion and α-ADR blockade on circulating granulocyte and monocyte numbers in humans *in vivo*. This is a novel finding in humans, and concurs with two animal studies also suggesting a potential mediating role of α-ADR for granulocyte increases despite methodological differences (40, 41).

Notably, because α-ADR blockade did not completely inhibit NE infusion-induced increases in neutrophil and monocyte counts, β-ADR pathways may also be involved in regulating changes in counts of these immune cells. So far, catecholamine infusion studies investigating β-ADR mechanisms suggest that β-ADR account for decreases or no effect rather than increases in numbers of circulating myeloid cells of the innate immune system. More precisely, β-ADR *agonist* infusion resulted in no change or lower numbers of circulating granulocytes and monocytes in humans (30, 33). In line with this, *in vivo* co-administration of β-ADR blockers and NE or EPI, which enhance α-ADR activation, led to increased circulating neutrophil numbers in rats (61) but had no effect in humans (62). Overall, comparing other studies with our paradigm is limited because of different methodologies regarding substance amounts, infusion and sampling periods, and the lack of control for plasma volume changes (30-32, 36).

Furthermore, catecholamine applications in the supraphysiological range are difficult to interpret, since high concentrations of e.g. NE can disproportionally activate β-ADR and thereby override α-ADR effects (63), or even induce α-ADR desensitization (64).

We can only speculate about the origin of the observed cell number increases. The short duration of the stressor or the NE-infusion duration, respectively, make *de novo* cell differentiation processes as seen after chronic stress exposure (65) unlikely. Thus, mobilization effects on mature innate immune cells seem more likely. α-ADR expression is highly dynamic during the lifespan of innate immune cells and is inducible by ADR agonists (66). We, therefore, assume that via α-ADR mechanisms NE can induce mobilization of mature myeloid immune cells transiently attached to endothelia in different organs framing the *marginal pool* (67, 68). Alpha- and β-ADR are expressed on myeloid immune cells, including neutrophils, monocytes, and macrophages (42, 43, 69), as well as on endothelial cells (70-72). Whereas previous research supports loss of cell-cell connection via β-ADR activation (62, 73, 74), our data also suggests an α-ADR mechanism to be involved in the observed increase of circulating cell numbers. Indirect α-ADR effects on myeloid immune cell numbers may result from increases in venous tone, peripheral resistance, and blood filtration causing hemoconcentration (56, 75). In line with the latter, we indeed observed that a significant difference in monocyte increases between saline+saline and phentolamine+NE disappeared after correcting for hemoconcentration.

4.4 Potential functional and clinical significance of stress-related increases in circulating numbers of monocytes and neutrophils

The potential functional and clinical significance of the observed acute stress-related transient increases in circulating absolute numbers of monocytes and neutrophils should be considered.

Neutrophils and monocytes are the first line of cellular defense against pathogens, and under non-stress or basal conditions only a part of these innate immune cells are patrolling in the blood stream (76). Absolute cell counts represent the detectable amount of the respective effector cells in the circulation and increases in innate immune cell counts represent more immune competent effector cells per blood volume unit and thus a greater effector cell concentration. If cell activity is not compromised, this may come along with a greater immune defense potential, at least transiently. Notably, increases in absolute cell numbers do not necessarily relate to cell percentage increases as the latter imply altered cell distribution but do not point to cell count increases or higher concentration of the respective cell types (compare Supplemental Tables S1 and S2, Supplemental Digital Content 4, http://links.lww.com/PSYMED/A495). In the sense of an unspecific immune reaction to acute stress (77), transient increases in circulating numbers of monocytes and neutrophils may reinforce the patrolling cells in the blood stream or traffic into organs to react faster to pathogens or tissue damage (e.g. (78)). This enforced immune reactive potential may represent a clear evolutionary benefit in acute injury situations, such as physical encounters or upcoming surgeries, where immune enhancement accelerates healing. However, in other stressful situations, an increased immune preparedness may potentially have clinical consequences (e.g. in transplantation surgery where immune suppression is warranted). Particularly, in vulnerable persons, such as patients with cardiovascular disease or other inflammatory diseases, acute increases in monocyte and neutrophil counts and related activity may trigger disease events such as acute coronary syndromes (79, 80). In addition, repetitive increases in numbers of innate immune cells due to repeated or chronic stress may, according to the model of allostatic load (26), accumulate over time and result in elevated basal monocyte and neutrophil numbers (65). Moreover, because effector functions of monocytes and neutrophils

include cytokine production, (22, 25) elevated numbers of these cells may induce a systemic low-grade inflammatory state having adverse health consequences, including cardiovascular disease (e.g. (27)).

4.5 Strengths, limitations, and conclusion

The major strength of our studies is the combination of cross-sectional with specific causality *in vivo* assessment in humans. We applied a NE-infusion design that mimics physiological NE-stress reactivity in terms of duration and dosage in an appropriate sample size and with appropriate Latin square trial balancing sequence. We controlled for hemoconcentration in both studies. Since neither NE (81) or phentolamine (82) cross the blood brain barrier, there is no interference with central-nervous effects in the infusion study and we interpret our findings as peripheral. We can exclude EPI as a confounding factor in NE infusion-induced cell number increases, as EPI levels did not concomitantly increase following NE-infusion. Our studies also have some limitations. We infused the same NE-dosage in all participants, which can increase interindividual variability of the immune cell number reactivity and thus the risk of non-significant findings. The risk of family-wise Type I error due to multiple testing cannot be excluded. After NE infusion, we measured about five times higher NE plasma concentration increases than after TSST exposure. However, during the infusion study we did not observe EPI increases excluding concomitant activation of the SAM axis and resulting additional release of NE from sympathetic nerve endings. As indicated by blood pressure increases from baseline to TSST performance (stress study: SBP: 13.37±23.82 mmHg, DBP: 11.69±17.07 mmHg) or from 10 min before infusion begin to maximum values during NE infusion (infusion study trial-2: SBP: 10.38±9.85 mmHg, DBP: 4.76±6.53 mmHg), the net efficiency of NE is slightly lower in

the infusion study, suggesting successful NE application within the physiological stress response range. Future studies are needed to replicate these findings in women and other populations, to address ADR mechanisms beyond non-selective α-ADR blockade, to clarify the origin and subset of the observed cell number increases, and to investigate the clinical relevance of the transient increase in innate immune cells.

In summary, our findings suggest that the mechanisms underlying stress-induced immediate increases in neutrophil and monocyte numbers in humans involve NE and at least in part α-ADRs. From an evolutionary perspective, this mechanism concurs with the fight-or-flight-paradigm and could have been helpful for survival after potential tissue damage. Increased stress-induced patrolling of myeloid innate immune cells, notably with phagocyting features (22, 25), likely increase the immune defense, thereby counteracting pathogen invasions and infections.

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Figure 1

Blood cell counts (means ± SEM) for monocytes and neutrophil, eosinophil, and basophil granulocytes before and 1, 20, and 60 min after the Trier Social Stress Test (TSST, grey area). Stress induced significant increases in all types of granulocytes (neutrophils: $p<.001$, eosinophils: $p=.019$; basophils: $p=.024$), and monocytes ($p<.001$) with highest cell counts immediately after stress. Whereas correction for hemoconcentration did not affect the stress response of monocyte and neutrophil cell counts, changes in eosinophil and basophil cell counts were reduced.

Figure 2

Blood cell counts (means ± SEM) for monocytes (Figure 1A) and neutrophil (Figure 1B), eosinophil (Figure 1C, and basophil (Figure 1D) granulocytes before, and 1, 10, and 30 min after infusion of either saline-saline (trial 1: Sa-Sa), saline-norepinephrine (trial 2: Sa-NE), or phentolamine-norepinephrine (trial 3: Ph-NE). Monocyte counts transiently increased in trials 2 and 3 as compared to trial 1 with highest increases in trial 2. Infusion reactivity of neutrophil counts differed between all trials with highest transient increases in trial 2. There were no significant trial differences across all time-points in eosinophil and basophil counts, although eosinophil counts increased acutely in trial 2.

Figure 1

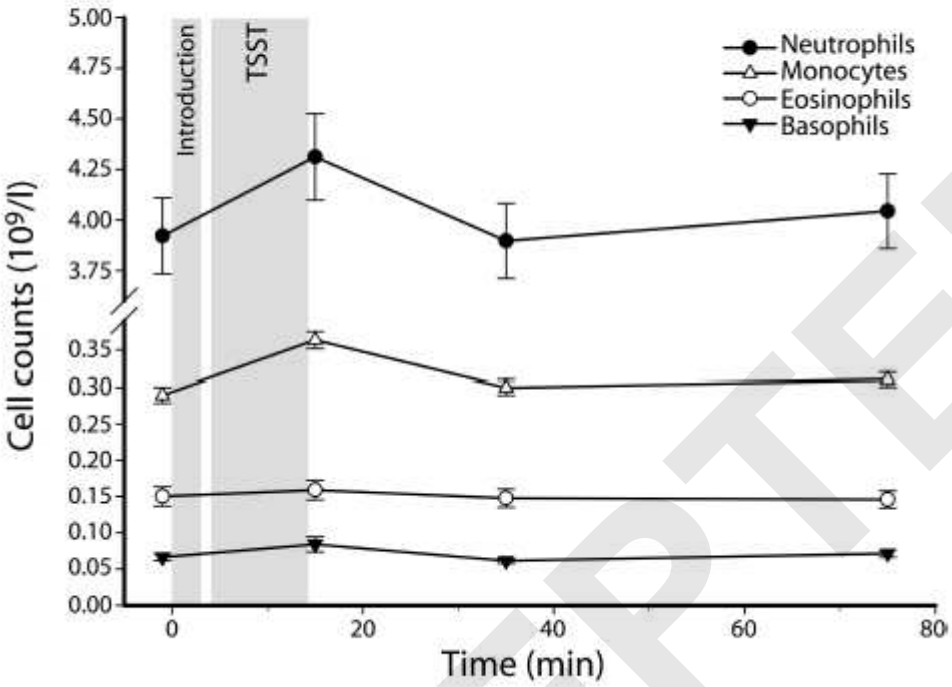

Figure 2 (panel A and panel B)

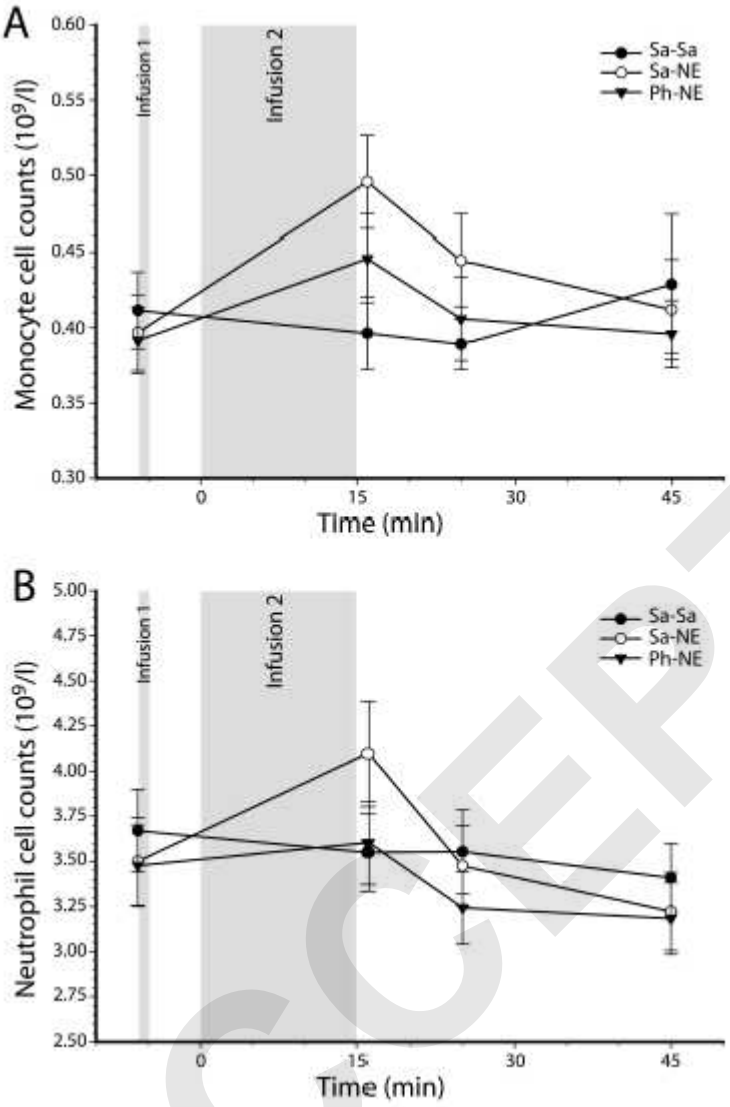

Figure 2 (panel C and panel D)

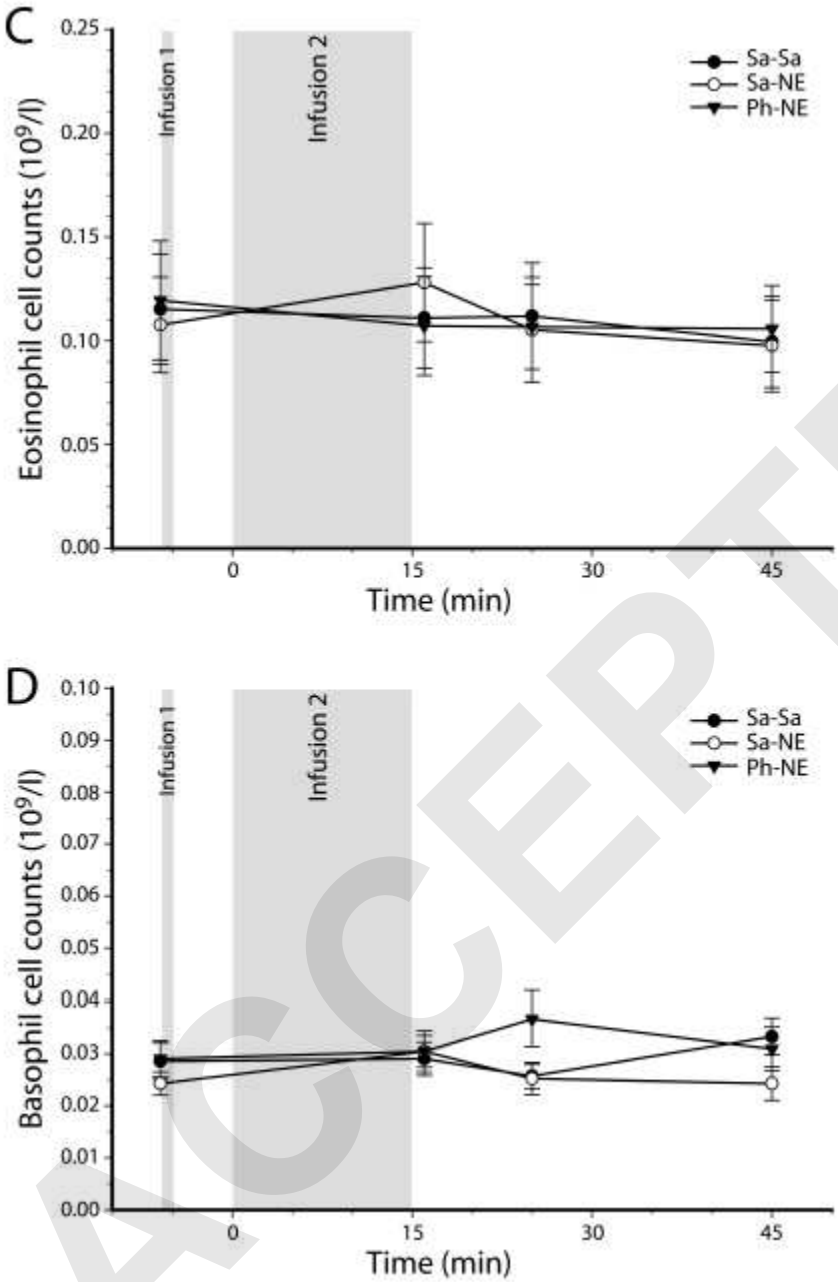

Table 1

Participants´ characteristics

Study	**Parameter**	**Mean ± SD**	**Range**
Stress-study N = 44	Age (years)	43.3 ± 13.8	21 - 65
	BMI (kg/m^2)	25.9 ± 3.0	20.7 - 34.3
	MAP (mmHg)	101.8 ± 12.3	82.8 -131.6
Infusion-study N = 21	Age (years)	53.3 ± 10.7	29 - 66
	BMI (kg/m^2)	24.0 ± 2.1	20.7 - 29.0
	MAP (mmHg)	92.2 ± 5.1	83.5 - 100.3

Table 2

Baseline measures (i.e. immediately before the respective treatment) of blood cell counts and catecholamine concentrations, and manipulation-induced changes in catecholamines and plasma volume

	Stress study	**Infusion study**					
		Trial 1 (Sa/Sa)	Trial 2 (Sa/NE)	Trial 3 (Ph/NE)	*Trial differences*		
					$p^{(1vs.2}$	$p^{(2vs.3)}$	$p^{(1vs.3)}$
Granulocytes baseline (10^9/l)							
Neutrophils	3,92±1,25 (1.84 – 7.44)	3.67±1.05 (2.41 – 6.49)	3.50±1.11 (1.98-6.60)	3.48±1.03 (2.13 – 5.90)	.28	.96	.22
Eosinophils	0,15±0,09 (0.02 – 0.36)	0.12±0.12 (0.00 – 0.45)	0.11±0.11 (0.00 – 0.48)	0.12±0.13 (0.01 – 0.54)	.54	.55	.77
Basophils	0,07±0,03 (0.02 – 0.16)	0.03±0.02 (0.01 – 0.07)	0.02±0.01 (0.01 – 0.04)	0.03±0.02 (0.00 – 0.08)	.13	.10	.86
Monocytes baseline (10^9/l)	0,29±0,07 (0.16 – 0.43)	0.41±0.12 (0.27 – 0.79)	0.40±0.11 (0.28 – 0.71)	0.39±0.10 (0.27 – 0.64)	.33	.89	.30
NE baseline (pg/ml)	366.51±124.14 (166.4-650.7)	416.98±227.31 (190.20 – 976.51)	388.63±238.47 (145.61 – 1097.49)	364.70±169.85 (126.10 – 778.06)	.46	.58	.18

EPI baseline (pg/ml)	40.65±19.06 (13.1 – 92.5)	30.92±18.69 (6.00 – 66.01)	29.29±15.99 (6.00-58.65)	26.17±11.88 (6.00 – 49.13)	.67	.40	.26
NE change (pg/ml)	156.13±88.62 (14.50 – 418.10)	7.71±88.33 (-183.35 – 281.97)[+]	870.05±430.63 (362.28 – 1858.21)	835.53±329.42 (376.08 – 1661.87)	**<.001**	.79	**<.001**
EPI change (pg/ml)	12.03±16.76 (-21.50 – 80.50)	2.08±11.57 (-20.14 – 37.51) [++]	-4.70±6.16 (-12.93 – 11.20)	0.97±10.36 (-10.23 – 36.42)	**.013**	**.03**	.75
Plasma volume percentage change (%)	-4.95±4.80 (-12.69 – 12.67)	1.60±3.25 (-7.98 – 6.49)	-3.41±3.19 (-11.04 – 1.27)	-1.34±2.43 (-6.09 – 3.82)	**<.001**	**.005**	**<.001**

Notes. Values are given as means ± SD (range). Change scores are calculated as post- (i.e. 1 min after the respective treatment) minus baseline measurements. Post-hoc tests of general linear models with repeated baseline or change measures were conducted to test for trial differences in baseline values and change scores. Bold values indicate significance. Sa, saline; Ph, phentolamine; EPI, epinephrine; NE, norepinephrine. [+] n=20, [++] n=19.